



Research review paper

Detecting un-authorized genetically modified organisms (GMOs) and derived materials

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ARTICLE INFO

Available online 3 February 2012

Keywords:

Un-approved GMO

Unknown GMO

GMO screening

Matrix approach

Genetically engineered organism

GEM

Un-approved GEM

ABSTRACT

Genetically modified plants, in the following referred to as genetically modified organisms or GMOs, have been commercially grown for almost two decades. In 2010 approximately 10% of the total global crop acreage was planted with GMOs (James, 2011). More than 30 countries have been growing commercial GMOs, and many more have performed field trials. Although the majority of commercial GMOs both in terms of acreage and specific events belong to the four species: soybean, maize, cotton and rapeseed, there are another 20+ species where GMOs are commercialized or in the pipeline for commercialization. The number of GMOs cultivated in field trials or for commercial production has constantly increased during this time period. So have the number of species, the number of countries involved, the diversity of novel (added) genetic elements and the global trade. All of these factors contribute to the increasing complexity of detecting and correctly identifying GMO derived material. Many jurisdictions, including the European Union (EU), legally distinguish between authorized (and therefore legal) and un-authorized (and therefore illegal) GMOs. Information about the developments, field trials, authorizations, cultivation, trade and observations made in the official GMO control laboratories in different countries around the world is often limited, despite several attempts such as the OECD BioTrack for voluntary dissemination of data. This lack of information inevitably makes it challenging to detect and identify GMOs, especially the un-authorized GMOs. The present paper reviews the state of the art technologies and approaches in light of coverage, practicability, sensitivity and limitations. Emphasis is put on exemplifying practical detection of un-authorized GMOs. Although this paper has a European (EU) bias when examples are given, the contents have global relevance.

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Abbreviations: Bar, (bialaphos resistance) gene encoding a phosphinothricin acetyltransferase (PAT), derived from *Streptomyces hygroscopicus*; Bla, gene coding for bacterial resistance to beta-lactamase antibiotics; BCH, Biosafety Clearing House; CaMV, Cauliflower Mosaic Virus; *cp4-epsps*, gene encoding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), derived from the common soil bacterium *Agrobacterium tumefaciens* strain CP4 and providing tolerance to glyphosate herbicides in plants; CRM, certified reference material; EC, European Commission; EFSA, European Food Safety Authority; ENGL, European Network of GMO Laboratories; EU, European Union; EURL-GMFF, EU's Reference Laboratory for Genetically Modified Food and Feed; HGE, Haploid Genome Equivalent; ISB, Information Systems for Biotechnology; ISK-class, Insert Sequence Knowledge class; LLP, Low Level Presence; LOD_{abs}, LOQ_{abs}, LOD_{pract}, LOQ_{pract}, absolute (copy number based) limit of detection/quantification, practical (sample specific, relative in per cent) limit of detection/quantification; MRPL, Minimum Required Performance Limit; *NptII*, (neomycin phosphotransferase II) gene coding for resistance to neomycin antibiotics; NRL, National Reference Laboratory; OECD, Organisation for Economic Co-operation and Development; P35S, 35S promoter (from CaMV); *Pat*, gene encoding a phosphinothricin acetyltransferase (PAT), derived from *Streptomyces viridochromogenes*; P-nos, nopaline synthase gene (*nos*) promoter from *Agrobacterium tumefaciens*; RASFF, Rapid Alert System for Food and Feed; T35S, 35S terminator (from CaMV); T-nos, *nos* terminator (see P-nos).

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Contents

1.	Introduction	1319
1.1.	Significance of GMOs	1319
1.2.	Genetic and phenotypic detection of GMOs	1319
2.	The “modular approach” in GMO analysis	1320
2.1.	The concept of modularity	1320
2.2.	Modules for GMO detection	1320
2.2.1.	Taxon specific modules	1320
2.2.2.	Element specific modules	1321
2.2.3.	Construct specific modules	1321
2.2.4.	Event specific modules	1321
2.2.5.	Validation and other modules	1322
3.	Classification of GMOs	1322
3.1.	Classification based on origin of the inserted genetic elements—four generations of GMOs	1322
3.1.1.	The first generation—single trait transgenes	1322
3.1.2.	Second generation—stacked trait GMOs	1322
3.1.3.	Third generation—near-intragenics	1322
3.1.4.	Fourth generation—intragenics and cisgenics	1322
3.2.	Classification based on DNA sequence information knowledge	1322
3.2.1.	Fully characterized GMOs (ISK-class 1)	1322
3.2.2.	GMOs not fully characterized but transformed with known construct(s) (ISK-class 2)	1323
3.2.3.	GMOs transformed with constructs containing genetic elements from GMOs of ISK-class 1 but where the specific construct is different from the GMOs of ISK-class 1 (ISK-class 3)	1323
3.2.4.	GMOs transformed only with genetic elements not used in GMOs of other ISK-classes (ISK-class 4)	1323
3.3.	Legal classification of GMOs	1323
4.	Where do un-authorized GMOs come from?	1324
5.	The matrix approach—the testing paradigm of today	1325
6.	Qualitative screening	1326
7.	Exploiting quantitative information	1327
8.	Exploiting traceability data and publicly available information	1327
9.	Reference materials	1327
10.	Analytical detection of un-authorized GMOs in practice	1328
10.1.	Direct detection of a registered, un-authorized GMO	1328
10.2.	Limited screening-based GMO testing of products consisting of a single or a low number of ingredients	1328
10.3.	Complex screening-based GMO testing of products containing multiple ingredients and/or where DNA quantity and/or integrity are significantly affected by processing	1329
11.	Alternative and advanced technologies for routine and ad hoc application	1330
11.1.	Fingerprinting techniques	1331
11.2.	Microarray technologies	1331
11.3.	Next generation sequencing technologies	1331
11.4.	Prospective of alternatives to PCR	1332
12.	Discussion and conclusion	1332
	Acknowledgments	1333
	References	1333

1. Introduction

1.1. Significance of GMOs

Genetically engineered or genetically modified (GM) plants, hereafter referred to as GM organisms (GMOs), are defined as organisms “in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination” (European Commission, 2001). As from the early developments of genetic engineering, the scientific society raised its concerns about this novel technology (Berg et al., 1975). As such, the development and in particular any commercial use of GMOs are subject to strict legal regulations in most countries around the world. The established legal framework mainly addresses all risks for the release of GMO in the environment and for consumption of GM materials for human and animal health. There can also be limitations to the use; a GMO can for example be legally used as a feed but not as a food. This was for example the case for StarLink™ maize in the USA. To support the freedom of choice of consumers, in many countries a mandatory or voluntary labeling of products containing GM material has been established, including specifications of thresholds for labeling.

Infringement of the legislation may lead in the worst case to the recall of a GM product from the market, resulting in major economic loss and/or ecological damage. It can also, hypothetically be necessary to retract a GMO after release. There is consequently a need for tools to detect, identify and quantify GMOs and products derived from GMOs. In the following the term GMO will include the derived products.

1.2. Genetic and phenotypic detection of GMOs

Almost all GMOs developed so far have been modified by insertion of extra DNA (BCH, 2011; CERA, 2011; GMO Compass, 2011). If all added DNA is derived from the recipient species itself or a species with which it can naturally exchange genes, then the resulting GMO is here classified as cisgenic if the inserted elements have not been rearranged, or as intragenic if the inserted elements have been rearranged (see also Lusser et al., 2011). If some or all added DNA is derived from another species with which the recipient cannot naturally exchange genes, then the resulting GMO is classified as transgenic (see also annexes of European Commission, 2001).

The genetic modification in all GM plants is, with the definition of GMOs above, by definition a change in the DNA sequence information (cf. Lusser et al., 2011). A genetic modification is by definition therefore always detectable at the DNA level. The presence of a genetic modification can often be detected at the transcriptional level, either due to synthesis of novel transcripts or indirectly by the silencing of native transcription (acting via RNA interference [RNAi]; mainly in in-tragenics and cisgenics; Frizzi and Huang, 2010). However, transcription regulation can be heavily influenced by external conditions among others the age/developmental stage of the organism, the organ/tissue/cell-type and temporal (circadian, seasonal) influence. Modifications affecting only the level (up or down regulation) of expression of genes already expressed can be particularly difficult to detect (see also Mukherji et al., 2011). Detection of GMO through transcription analyses is therefore not commonly applicable and requires in general more scrutiny against false positives/negatives than targeted DNA sequence analyses.

Translational products, i.e. proteins, have also been used as GMO tagging targets. Distinct transcripts are sometimes translated into indiscriminate proteins due to the degeneracy/redundancy of the genetic code. Because of this and their inherent dependence on transcription and their generally lesser stability, proteins are not suitable for GMO detection in a wide range of products (e.g. processed food/feed).

Finally, the expressed phenotype itself can sometimes provide sufficient information to identify a GMO, e.g. when the novel trait provides tolerance to a particular herbicide (www.seedtechnology.net). Again, the range of application of the phenotype as a GMO detection tool is very limited. In case of herbicide crop tolerance, the plants need to be grown. Also, distinction needs to be made between engineered phenotypes and naturally occurring mutants. Phenotypic approaches are therefore generally not very suitable for detection/identification of a GMO.

2. The “modular approach” in GMO analysis

2.1. The concept of modularity

In the EU and many other countries, the GMO analytical procedure is viewed as the complete procedure starting with a sample and including all steps performed to determine the presence, identify and quantify (when necessary) the GMOs in products such as food, feed or seed, until finally a measurement result is provided (Fig. 1).

An example of this principle can be found in the guidance document on measurement uncertainty for GMO testing laboratories adopted by the European Network of GMO Laboratories (ENGL) (see Fig. 2 in Trapmann et al., 2009). It is also reflected in the organization of the international standards on GMO detection (see Fig. 1 in ISO,

2006). A central component of all analytical procedures is the analytical method itself. As for GMO detection in the EU, DNA is to date the sole analyte applied for legal purposes (European Commission, 2004a; Holst-Jensen et al., 2006). An analytical procedure for GMO testing covers the sample preparation, DNA extraction and purification, (real-time) PCR amplification and data evaluation. Historically, the analytical method was considered an indivisible unity, with reference to a phenomenon called a “matrix effect”. The matrix effect in analytical chemistry is defined as: “the combined effect of all components of the sample other than the analyte on the measurement of the quantity” (IUPAC, 1997). However, there is now a growing acceptance of the so-called “modular approach” (Holst-Jensen and Berdal, 2004), provided that specific performance requirements are satisfied (see e.g. Bellocchi et al., 2010; Debode et al., 2007; European Commission, 2006b; Grohmann et al., 2009; 2011). The modular approach has also recently been adopted for microbiological applications (Kagkli et al., 2011). According to the modular approach the sample preparation, DNA extraction and PCRs for individual target sequences can be treated as separate modules that together form a method. A module can therefore be defined as a distinct and limited operation that is performed on an input material and which delivers an altered output material or data. Examples of modules are: 1) a sample preparation module where the input material is e.g. grains and the output material could be flour; 2) a DNA extraction and purification module where the input material is flour and the output material is purified DNA in aqueous solution; 3) a real-time PCR module where the input material is a purified DNA in aqueous solution and the output material is a measurement of fluorescence and translation into a number of target sequence copies; or 4) a data evaluation module where the available data are processed into a final measurement result (Fig. 1).

2.2. Modules for GMO detection

The advantages of a modular approach are primarily increased flexibility and a potential for ad hoc design of rational and cost effective validation and testing strategies. The modular approach has so far mainly found application for analyses of DNA using PCR technology, but the approach is theoretically applicable also to other targets than DNA. Here, we will focus only on the PCR module, which could be recognized as the analytical module *sensu stricto* in GMO analysis.

Each analytical module is characterized first of all by its specificity. For PCR modules it is common to discriminate between four levels of specificity (Fig. 2) in the context of GMO detection.

2.2.1. Taxon specific modules

A species/taxon specific module detects a sequence known to be specific for the target species/taxon. Ideally the target sequence is

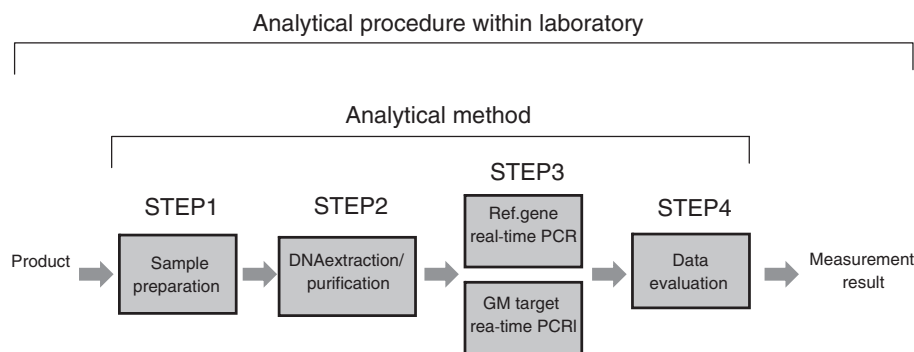


Fig. 1. The modular approach in GMO analysis. The analytical procedure within the laboratory is shown, but the complete analytical procedure also includes the sampling taking place upstream. The analytical method is a core element in the analytical procedure and is viewed as a series of steps, each involving the use of one or more modules (boxes). Each module is a distinct tool or operation. For a single step there are sometimes several alternative modules available, allowing for increased flexibility. Modified from Holst-Jensen and Berdal (2004) and Trapmann et al. (2009).

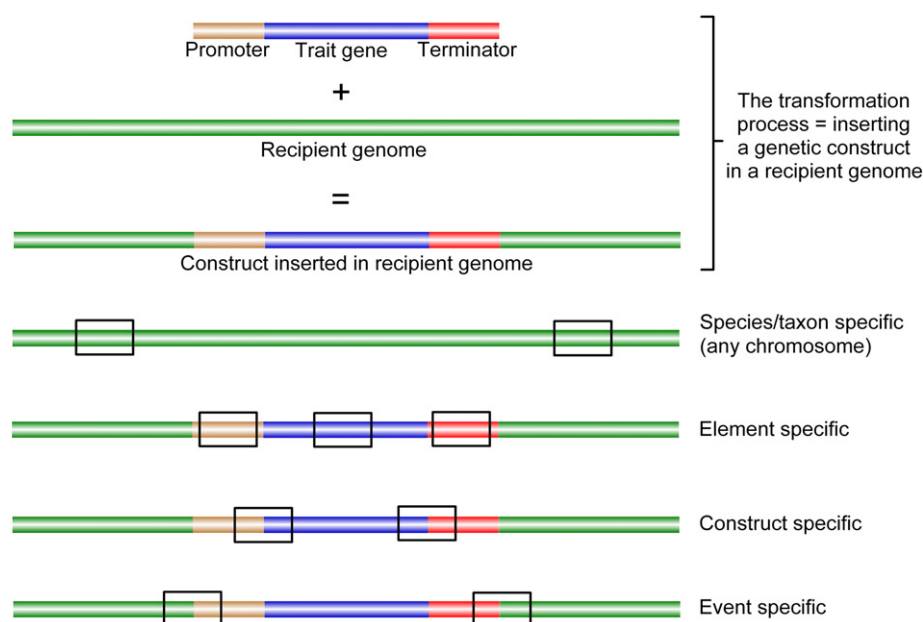


Fig. 2. The transformation process and the four levels of specificity of analytical modules targeting DNA sequences. In some cases there are additional partial or complete constructs inserted. These can be located at the same or other loci (BCH, 2011; CERA, 2011; GMO Compass, 2011). The typical species/taxon specific screening target is a single copy seed specific or housekeeping gene.

consistently present in the target species/taxon and absent in other taxa. Furthermore, it should not exhibit allelic variation (Hernandez et al., 2004; Papazova et al., 2010). In case the module is intended for quantitative purposes, presence of the target sequence as a single copy in the haploid genome of the taxon is preferred. Species/taxon specific modules are suitable for identification and quantification of ingredients (defined as all material derived from a given species/taxon). The measured quantity of species/taxon material in a product provides the basis for calculation of the relative GMO content per ingredient, as for example required in EU regulations (European Commission, 2003b; 2011). This quantity is most accurately estimated with reference to haploid genome equivalents (HGE) as recommended by the EC (European Commission, 2004a; see also Holst-Jensen et al., 2006). It is usually necessary to determine the limit of detection (LOD) and the limit of quantification (LOQ) of a PCR module. These parameters are initially determined in absolute terms (number of units of the analytical target) using a single ingredient (absolute LOD [LOD_{abs}] and LOQ [LOQ_{abs}]). The combined data for the two PCR modules are often translated into a “PCR method” specific relative LOD and LOQ. In real-life situations ingredients are often mixed and/or the ingredients have been processed in ways that reduce the quantities and detectability of target DNA sequences present in a product. The true relative LOD (and LOQ) will therefore differ between samples. The species/taxon quantity is the main parameter determining the practical (sample specific) relative limits of detection (LOD_{pract}) and quantification (LOQ_{pract}) for GMOs belonging to that particular species/taxon (cf. Holst-Jensen et al., 2003).

2.2.2. Element specific modules

A single element specific PCR module targets a single discrete inserted DNA sequence motif. This motif can for example be a promoter, a terminator, an intron or the coding part of a gene. Element specific modules are suitable for screening to determine whether a sample is likely to contain GMO or not. Element screening can also limit the list of candidate GMOs and provide clues to the identity of present GMO(s). Both regulatory elements such as promoters and terminators, as well as trait and selection marker genes and cloning vector elements can be targeted with single element specific PCR modules. The majority of single elements are to date derived from

natural (non-GM) donors such as viruses, bacteria and plants. Thus, the presence of single elements does not always provide conclusive evidence of presence of GMO. In many cases it is therefore necessary to include as a control a specific PCR module to test for the presence of the donor organism. These control modules would belong to the taxon specific modules (Section 2.2.1).

2.2.3. Construct specific modules

A construct specific PCR module targets an inserted DNA sequence motif composed of at least two elements that do not naturally co-exist in this conformation. In other words, in such a chimeric sequence the 5' and 3' end of the sequence motif are derived from two separate elements. A construct specific module applies two discrete oligonucleotide primers each hybridizing to one of the elements in the chimera. Construct specific modules are suitable for screening and quantification and provide indisputable evidence of presence of GMOs when a positive test result is obtained. Construct specific modules are only exceptionally suitable for identification of a GMO since the same construct (or chimeric motif) can be present in more than one GMO.

2.2.4. Event specific modules

Finally, an event specific PCR module targets a sequence motif unique to a single GMO. The event specific sequence motif is the unequivocal signature, or tag, of a particular GMO. In most cases it is created de novo when the construct is integrated into the recipient genome. Event specific sequence motifs are usually but not always the integration-border regions i.e. the fusion sequence composed of the terminal basepairs of the inserted DNA and the adjacent basepairs of the recipient host genome at the insertion locus. A construct specific target (Section 2.2.3) cannot be event specific because the same construct can be used repeatedly to develop new GMOs. Event specific PCR modules are particularly suitable for identification and quantification and represent the legal basis in the authorization of a GMO for commercial use as food/feed in among others the EU. To approve a GMO for commercial use, the EU requests the notifier to provide a detection method specific to the GMO and the corresponding control samples (European Commission, 2003a). The provided method is then validated through interlaboratory testing under supervision of

the EU Reference Laboratory for GM Food and Feed (EURL-GMFF) assisted by the ENGL. The results of these analyses together with the details on the method are published by the EURL-GMFF on its website <http://gmo-crl.jrc.ec.europa.eu/>.

2.2.5. Validation and other modules

Multi-laboratory and collaborative trial validations of GMO detection methods are also conducted outside the EU legal context. For example the validation of a DNA extraction module (Waublinger et al., 2007), and of real-time PCR modules for species specific (Yang et al., 2005), element and construct specific (Feinberg et al., 2005; Grohmann and Mäde, 2009; Grohmann et al., 2009; 2011; Shindo et al., 2002), and event specific targets (Akiyama et al., 2010; Pan et al., 2007). One multiplex assay has also been collaborative trial validated: the DualChip® GMO (Leimanis et al., 2008). Informative sources on the status of some representative GMO detection methods include but are not limited to the GMO detection methods databases (GMDD; Dong et al., 2008; <http://gmdd.shgmo.org/>) and (GMO-METHODS; <http://gmo-crl.jrc.ec.europa.eu/gmomethods/>; EURL-GMFF, 2011).

3. Classification of GMOs

3.1. Classification based on origin of the inserted genetic elements—four generations of GMOs

3.1.1. The first generation—single trait transgenes

The first generation and most of the present commercial GMOs were produced using enzymatic cut-and-paste technology (restriction digests and ligation). In these GMOs the desired trait gene(s) were combined with virtually universally functional and constitutive promoters and terminator elements such as the cauliflower mosaic virus (CaMV) 35S promoter (P35S) and terminator (T35S) and the nopaline synthase promoter (P-nos) and terminator (T-nos) derived from *Agrobacterium tumefaciens* (Hemmer, 1997). Frequently a second construct was also made that would typically code for a phenotype that facilitated identification and selection of the transformed plant cells. These genetic constructs were then inserted into circular cloning vectors of bacterial or viral origin for rapid propagation.

The cloning vectors usually also contained marker genes for selection and propagation of those bacteria in which the vector had been transfected. The specific insertion locus of the construct into the recipient plant genome varied from cell to cell, and it was the rule that some parts of the cloning vectors in addition to the desired construct(s) were co-inserted. Thus, the first generation and most of the current commercial GMOs contain members of a limited set of genetic elements.

The functional genetic construct responsible for a desired trait contains at least a promoter, a gene and a terminator. Constructs often also contain an additional enhancer, intron or signal motif, contributing to transcriptional regulation and post-transcriptional signaling in the cell. Cloning vector derived elements such as selection marker genes, polylinkers and the *Agrobacterium* Ti-plasmid left and right border elements are present in many but not all GMOs. The presence of these elements and their specific distribution, combinations and organization in different GMOs represent information that can be exploited by analysts. We will return to this in Sections 5 and 10.

3.1.2. Second generation—stacked trait GMOs

The second generation of GMOs, i.e. the so-called stacked GMOs are usually hybrid crosses between first generation GMOs (e.g. Bt11×GA21 maize) or retransformed first generation GMOs (e.g. MON15985 cotton, retransformed from MON531 cotton). Stacked hybrid GMOs are difficult to discriminate from their parental first generation GMOs, with exception of testing of single seeds or plants

(Akiyama et al., 2005; Holst-Jensen et al., 2006; Taverniers et al., 2008). Many of the commercial cotton and maize GMOs grown at present are stacked GMOs (James, 2011) and nearly 50% of the dossiers received by the European Commission since 2004 concern stacked GMOs (GMO Compass, 2011).

3.1.3. Third generation—near-intragenics

The third generation of GMOs is comprised of so-called near-intragenics or GMOs where the inserted transgenic elements have not been used in other (known) GMOs. Near-intragenics are GMOs where the major part of the insert is host-derived and where the recombinant part of the insert is very restricted (e.g. limited to short segments derived from the cloning vector). The potato event AV43-6-G7 is an example of a near-intragenic GMO (AVEBE, 2009). Common to third generation GMOs is also that they are much more difficult to detect than first and second generation GMOs. This is discussed in more detail in Section 11.

3.1.4. Fourth generation—intragenics and cisgenics

True intragenics and in particular cisgenics, are likely to be introduced as a fourth generation of GMOs (Lusser et al., 2011). In these GMOs, the inserted elements will invariably be derived from the gene pool available for natural recombination for the recipient species. Thus, detection of the inserted elements alone cannot be used as evidence of genetic modification. Fortunately, even for these GMOs, the specific order and insertion loci of the inserted elements will offer potential for DNA based detection and identification. It is of particular relevance that typical event specific motifs associated with the insertion loci (cf. Fig. 2) are likely to appear also in these GMOs. Gene expression patterns also offer some, however limited potential for detection of intragenics and cisgenics, cf. the discussion of transcriptomics approaches in Section 1.2.

3.2. Classification based on DNA sequence information knowledge

The key to detection and correct identification by and large lies in knowledge about the inserted elements, their organization and the insertion loci flanking the inserts. It is therefore logical to classify GMOs based on insert sequence knowledge (ISK) available to the analytical laboratory developing or using the analytical module(s).

3.2.1. Fully characterized GMOs (ISK-class 1)

This class is comprised by GMOs where the complete insert and flanking DNA sequences are known. Within the EU a notifier applying for authorization of a GMO must provide this information along with an event specific quantitative detection method and control samples to the EC services (European Commission, 2003a, 2003b). ISK-class 1 includes all GMOs authorized for commercial release within the EU. However, for the National Reference Laboratories (NRLs) within the EU (European Commission, 2004b, 2006b) it also includes all other GMOs for which a complete dossier is available to the European Food Safety Authority's (EFSA's) GMO panel and the national Competent Authorities (e.g. via EFSA's GMO Extranet; <https://sciencenet.efsa.europa.eu/portal/server.pt/>). Other countries that require a specific detection method to be submitted together with an application for authorization include among others Brazil (Brazil, 2008), Malaysia (Malaysia, 2007) and South Africa (South Africa, 2005). GMOs pending authorization and GMOs for which authorization is not renewed (see e.g. European Commission, 2007a, 2007b, 2007c, 2007d, 2007e), are to be considered as ISK-class 1 GMOs. Detection and correct identification of ISK-class 1 GMOs can be done using event specific modules. Before authorization the EURL-GMFF together with members of the ENGL validates a specific detection method for the GMO. After validation the EURL-GMFF publishes all validated methods on the internet (<http://gmo-crl.jrc.ec.europa.eu/statusof doss.htm>). Corresponding certified reference material is to

be made available in parallel. All EU authorized and other GMOs for which the EURL-GMFF has validated a method can consequently be considered members of ISK-class 1 for all GMO laboratories in the world.

3.2.2. *GMOs not fully characterized but transformed with known construct(s) (ISK-class 2)*

The same genetic construct is often used to develop several transformants (events). Usually, only one or exceptionally a few of these events are brought as far as to field trials and commercialization. However, there are several examples of such sister or back-up events and in some cases only one of them is authorized. Examples include MON809 and MON810 maize, T14 and T25 maize, and RF1 and RF2 rapeseed (CERA, 2011; GMO Compass, 2011). There are even a few apparent examples where, due to human error and/or insufficient quality control at the breeding/seed handling, an un-authorized event escaped and was later traced in the food or feed chain: CBH351 (StarLink™) maize (EPA, 2008), Bt10 and Bt11 maize and DAS 59122 and DAS 59132 maize (see <http://gmo-crl.jrc.ec.europa.eu/>). For CBH351, Bt10 and DAS 59132 maize, event specific modules were not available by the time the GMO was first detected in the food/feed chain. This exemplifies that reliance only on the availability of event specific modules is incompatible with detection of un-authorized homologous events. Construct specific modules on the other hand are well suited for detection of such events, but are unfit for identification/discrimination between them.

Construct specific detection modules are ubiquitously designed to detect artificially fused genetic elements. Despite being referred to as “construct specific” these modules do not detect the full construct, only pairs of fused elements, so-called chimeras. ISK-class 2 therefore includes all GMOs where the fusion of elements within the construct is known a priori, even if the full length construct is not known. Some of the construct specific modules are well suited as screening modules for the detection of GMOs, see e.g. Waiblinger et al. (2010).

3.2.3. *GMOs transformed with constructs containing genetic elements from GMOs of ISK-class 1 but where the specific construct is different from the GMOs of ISK-class 1 (ISK-class 3)*

A few transcription regulators such as the CaMV P35S and T-nos have been used in a multitude of GMOs, including both commercial and purely experimental events. Some trait genes, in particular from the first generation of GMOs, have also been used quite frequently: e.g. *bar*, *cp4-epsps*, *cry1A(b)* and *pat*. However, these genes have commonly first been modified to optimize transcription and translation in eukaryotes and represent as such complete de novo artificial DNA sequences unique to GMO. A few selection marker genes are also commonly found in first and second generation GMOs, e.g. *nptII* and *bla* (CERA, 2011; GMO Compass, 2011; Hemmer, 1997). Several of these elements are derived from non-GM donor organisms that can be found in the environment and sometimes also in the food chain. For particular genetic elements allelic differences at the DNA sequence level are sometimes observed between different GMOs, and between the natural donor and GMO(s). Detection methods targeting these genetic elements are commonly exploited, successfully, to screen for presence/absence of GMOs, and to obtain useful information about the nature of putatively present GMOs (e.g. Chaouachi et al., 2008; Hamels et al., 2009; Novak et al., 2009; Van den Bulcke et al., 2010; Waiblinger et al., 2010). Allelic differences, in particular at the primer and probe binding sites of a PCR, can affect the detectability of a particular genetic element (Broothaerts et al., 2008; Ghedira et al., 2009; Holst-Jensen, 2009; Morisset et al., 2009). There are for example, substantial differences between the *cry1A(b)* gene variants found in the natural (non-GM) donor *Bacillus thuringiensis* ssp. *kurstaki*, and Bt11, Bt176 and MON810 maize (Fig. 3). The haplotypes of the gene in the three GM maize events have been differently optimized for expression in plants/maize. The CaMV P35S on the other hand is an

example of an element where significant allelic variation is also found naturally (Fernandez et al., 2005). Positive analytical signals can be caused by the natural non-GM donors and negative analytical results can be caused by allelic variation. Prudent interpretation of analytical results obtained with screening modules is therefore required. The use of a control module to detect the donor is strongly recommended in some cases. Such modules are unfortunately only exceptionally available (e.g. Cankar et al., 2005; Chaouachi et al., 2008 Wolf et al., 2000 [CaMV]; Co-Extra, 2009 [Agrobacterium, Figwort mosaic virus and *Bacillus thuringiensis*]).

3.2.4. *GMOs transformed only with genetic elements not used in GMOs of other ISK-classes (ISK-class 4)*

With the emergence of high-throughput DNA sequencing technologies, the availability of novel genetic information including functional characterization of a multitude of genes and transcription regulators from all kingdoms of life is growing at a high pace. For developers of GMOs this means that the selection of available genetic elements that can be combined and introduced to new GMOs is becoming virtually unlimited. For these elements there is usually no validated/reliable detection module available. With accumulating knowledge and technological progress it is also made easier to introduce small modifications down to single nucleotide changes in genetic elements found in GMOs of ISK-classes 1, 2 and 3. See Lusser et al. (2011) for a detailed review of novel plant breeding technologies of relevance. Such changes can be introduced and the result will then be that the element(s) cannot be detected with the detection modules suitable for GMOs of ISK-classes 1, 2 and 3. The novel GMO remains undetectable, even if an inserted element is putatively the same as one previously known. Within the EU, a GMO can change status from ISK-class 4 to ISK-class 1 once an application for EU authorization of that GMO is received, provided that the application is complete. Some of the GMOs currently pending authorizations within the EU are examples of GMOs that went from ISK-class 4 status (as unknown) to ISK-class 1 as a consequence of the authorization process: the soybean events BPS-CV127-9 and DP 305423 and the maize events LY 038 and DAS-40278-9. There are other GMOs in various stages of development and testing outside the EU that for the Competent Authorities in the EU de facto fall into ISK-class 4. Notably, this also demonstrates that occurrence of traces of a GMO of ISK-class 4 in the food chain within the EU is not an unlikely scenario. The situation is the same for other jurisdictions in the world because of the lack of an effective global channel for communication of relevant information on GMO developments and field trials (see also GAO, 2008 and Section 12).

3.3. Legal classification of GMOs

GMOs are either authorized (approved, de-regulated ...) and therefore legal or they are un-authorized (un-approved, regulated ...) and therefore illegal on the market. Presence of some GMOs for which authorization is pending or not renewed, is or has been tolerated for a limited period within the EU, provided that the quantity is below a specified threshold and that the presence is adventitious and technically unavoidable (European Commission, 2003a, 2007a, 2007b, 2007c, 2007d, 2007e). Regulation EC 619/2011 (European Commission, 2011) applies only to feed and provides details on the sampling and methods of detection for low level presence (LLP) of particular GMOs for which an authorization procedure is pending or the authorization of which has expired. The core of this regulation is the introduction of a minimum required performance limit (MRPL) of 0.1% (relative to mass) as “the lowest amount or concentration of analyte in a sample that has to be reliably detected and confirmed by official laboratories”. If the analyses cannot verify that the concentration exceeds the MRPL (taking the measurement uncertainty into

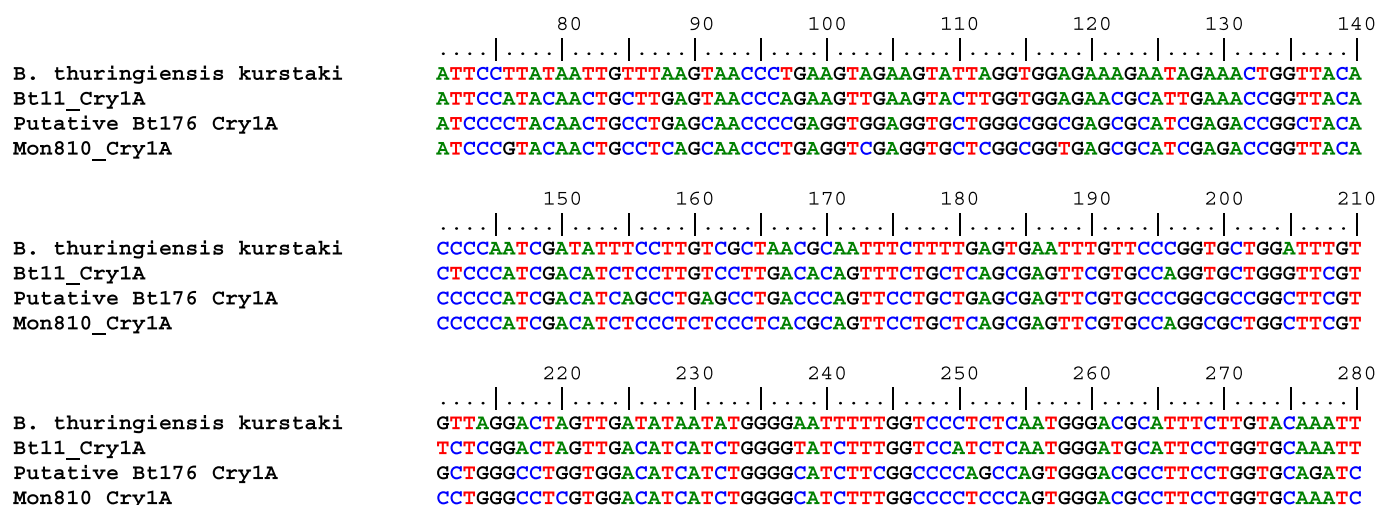


Fig. 3. Segment of *cryIA(b)* gene alignment for the native *Bacillus thuringiensis* ssp. *kurstaki* gene and three GM maize events Bt11, Bt176 and MON810, showing significant allelic variability. The full length gene is more than 1800 bp. The alleles found in Bt11, Bt176 and MON810 are modified to optimize their expression in plants/maize.

consideration) then the sample is to be considered in compliance with the regulation.

In other jurisdictions, other practices may be applied. In Norway for example, a European country that itself is not a member of the EU, no GMO has yet (January 2012) been authorized for cultivation or use in food or feed. But all GMOs authorized in the EU are in practice tolerated in food and feed in Norway, provided that the presence is adventitious and that the quantity does not exceed the EU threshold for labeling (0.9%). Presence of minor quantities of GMOs pending authorization may also be tolerated in some jurisdictions, under particular conditions. In case of legal infringement, the EU has put in place specific emergency measures to cope with particular unauthorized GM maize or rice events (European Commission, 2005, 2006a, 2008). Japan is an example of another country where emergency measures have also been implemented, e.g. for CBH351 and Bt10 maize (Watanabe et al., 2007 and references therein). In 2011 linseed (flax, event FP967), rice (LL601 and events expressing Bt and/or CptI protein), papaya (55-1 and PRSV-YK) and rape seed (event GT73 in *Brassica rapa*) are subject to inspection orders in Japan (JMHLW, 2011) based on a perceived high risk of violation of the Food Sanitation Law. These are examples of documents that can support decisions on sampling, testing and other actions to be taken. The enforcement authorities and other involved stakeholders therefore have to consult detailed legislation in cases where unauthorized GMO is detected or its presence is particularly likely.

4. Where do un-authorized GMOs come from?

One of the first incidents where an un-authorized GMO was detected concerned the maize event CBH351 (StarLink™). This GMO was authorized only for use in feeds but not in foods (EPA, 2008). The un-authorized GMOs found and reported are most commonly GMOs that are authorized in one jurisdiction but not in another (Busch et al., 2004; Ruttink et al., 2010b). It is difficult to exclude intermingling of small quantities of such GMO into exported shipments. Since the first field trials and commercialization of GMOs took place, the USA has been the global leader in the application of gene technology in plant breeding. Not surprisingly therefore, that un-approved releases and intermingling have been reported several times in the USA. The problem was reviewed by the US Government Accountability Office for both domestic and imported “un-authorized” GMOs (GAO, 2008). Global trade, incomplete sanitation and the complexity of segregating supply chains, all contribute to the difficulties. It is commonly acknowledged that a GMO authorized in one

jurisdiction is unlikely to represent a significant health or environmental risk in another jurisdiction, also if the GMO has not been authorized or even risk assessed in the second jurisdiction. However, exceptions can be imagined. For example 1) if the GMO is not sufficiently reproductively isolated from or is particularly likely to affect an endemic species in the country representing the second jurisdiction; 2) the GMO belongs to a species serving as a staple food source in the second jurisdiction but not in the first, and introduction of the GMO could result in unacceptably high levels of a GMO derived bioactive compound in the local diet in the second jurisdiction; or 3) particular abiotic conditions in the second jurisdiction could alter the performance of the GMO in an undesirable way.

A second possible source of un-authorized GMOs is escape from field-trial releases. There are several examples of such escapes, and sometimes these represent a significant risk to health and the environment. This was for example the case with maize events developed by the US company ProdiGene. The events developed to produce experimental pig vaccine were found as volunteers in soybean and sorghum fields on former GM maize test plots (APHIS, 2011; Fox, 2003). Another example is the release of glyphosate tolerant creeping bentgrass (a golf grass) by the Scotts Company LLC (APHIS, 2011; Zapiola et al., 2008). Failure in the quality control at the seed production level is another possible reason for un-authorized release of GMOs as exemplified with the Bt10 maize case. Use of trait specific ELISA tests that are unable to discriminate between an un-authorized and an authorized event significantly increases this risk compared to the use of event-specific PCR tests. Un-authorized release of GMO can often affect trade and the local economy (GAO, 2008). For example the 2006 incident of widespread low level contamination of rice produced in the U.S.A. with the regulated (un-authorized) event LL601 (Vermij, 2006) resulted in import bans for up to 2 years in the EU, Japan, Russia and South Korea. Analytical testing of rice and associated costs for the exporters and importers immediately boosted. The economic impact for the U.S. rice industry was significant but relatively short and the actual impact in U.S. dollars has been difficult to estimate (Li et al., 2010; Redick and Endres, 2009). A U.S. court recently recommended that Bayer CropScience pay 125 million US\$ in punitive damages and US\$ 11.8 million in compensatory damages (Fox, 2011). The investigators from the U.S. Animal and Plant Health Inspection Service had hoped to identify how each GM rice line entered the commercial rice supply, but the exact mechanism for introduction could not be determined in either instance (APHIS, 2011). Escapes from field-trials can be a particularly difficult problem in situations with poor communication on the GMO activities. In China, at

least three un-authorized GM rice events (namely Bt63 synonymous to TT51-1, KeFeng-6 and KMD1 synonymous to Kemingdao) emerged unexpectedly in the food supply chain. Information about these GMOs and material for characterization and development of suitable detection methods has only recently been obtained (see Reiting et al., 2010; Wang et al., 2011 and Wu et al., 2010 and references therein). Reports published on the European Rapid Alert System for Food and Feed (RASFF; <https://webgate.ec.europa.eu/rasff-window/portal/>) show that these rice events are not uncommon in products from China. The risk that people with access to un-authorized GMOs during development and field trials take seeds for own use or give away such seeds to others is not negligible. Furthermore, this risk is likely correlated with the perceived personal cost-benefit and negatively correlated with the educational level of the workers. The possibility that a GMO is escaping from field-trial releases into the environment and/or eventually ends up in the food supply chain without proper authorization therefore cannot be excluded. An escaped GMO can also end up in other countries than the country where the field-trial took place. International collaboration and open information channels distributing data on field releases are therefore imperatives to minimize the risk of negative consequences to health and the environment of such escapes. The U.S. ISB (<http://www.isb.vt.edu/>), the OECD BioTrack and the BCH databases are helpful in this respect (see e.g. Stein and Rodriguez-Cerezo, 2009), but all these information systems could be drastically improved.

5. The matrix approach—the testing paradigm of today

With the large number of species and GMOs that can be present simultaneously in many products in the food supply chain, it is evident that testing directly for presence/absence of each and every GMO is extremely labor intensive and costly. The use of initial screening for presence/absence of candidate species and elements common to multiple GMOs can facilitate rapid and cost efficient discrimination of samples: those containing GMO and those where no GMO can be detected. Such screening can simultaneously provide clues to the identity of present GMOs.

The EU-funded GMOchips project was first to conceptualize and systematically explore the consensus and matrix approaches (European Commission, 2010). The latter approach has since been further developed and is now applied in the majority of GMO testing laboratories around the world. It is clearly the prevailing GMO testing paradigm.

Setup and implementation of the matrix approach is a stepwise process. First a relational matrix is established (Fig. 4). This matrix is a table giving an overview of the presence/absence of analytical targets for a set of GMOs. For each analytical target, a corresponding analytical module must be available. Ideally, the presence/absence is experimentally verified for every combination of analytical module and GMO. The latter should be represented by a reference material from a reliable source, such as the developing company or a certification body. The verification is important because of possible allelic variation affecting the detectability, and errors and incompleteness in published data and documents describing each GMO.

The term matrix is here used in the mathematical sense referring to the relations between the two variables: the GMO and the analytical module detecting an element contained in the GMO (= the target of the module). It is not to be confused with the more common use of the term matrix to refer to a particular type of product subjected to analysis (e.g. sausages, flour, seeds...). The numbers of GMOs and specific tests included in the matrix are flexible, and can be increased or decreased according to the available information, needs and specific situations. The frequency and distribution of the targets of screening modules among GMOs of the same and/or different species can be exploited in the design and subsequent use of the matrix. The information can for example be used to design decision trees.

Building up a matrix with modules representing all the specificity levels (cf. Fig. 2) and experimentally verified data confirming the performance of each module in combination with each GMO is a huge task. Publicly available lists of field trials performed in various countries will usually contain limited information on the specific DNA sequences associated with the genetic modifications (Degrassi et al., 2003). However, the lists will normally include some information on the nature of the traits and possibly also on the sources of the trait genes. Literature surveys, databases and bioinformatics can then be combined in efforts to design screening tools for molecular detection of the GMOs.

International collaboration would clearly be beneficial. Indeed, much of the work performed by method developers around the world so far can be seen as duplicate work. There are for example multiple published single element screening modules targeting the CaMV P35S and T-nos. The target sequences and formats of these modules are in some cases clearly distinct. Several of these modules have also been collaborative trial validated against variable numbers of GMOs (see e.g. EURL-GMFF, 2011). On the other hand, for the majority of alternative promoters and terminators, cloning vectors or popular trait genes, there is presently no corresponding validated single element screening module.

In the successive steps, when implementing the matrix approach to analyses of samples, the matrix is used as a reference. The results from application of selected screening modules on the sample are compared with the data tabulated in the matrix. Observed presence/absence patterns matching those predicted for (a) particular GMO(s) are indicative of presence of the(se) GMO(s) in the sample. Putative presence can then be verified using more specific analytical modules such as event specific PCR modules. Notably, a satisfactory LOD_{pract} is required.

Many laboratories mainly do single species analyses, e.g. seed testing. In this case it may suffice to consider only the GMOs belonging to that single species and the related sub-matrix. If the product is unlikely to contain any other species, or if it for other reasons is reasonable to assume that presence of GMO is unlikely, it can be cost efficient to go directly to screening for GMO specific elements. If the GMO screen is positive then impurities from other species should be assessed to avoid interference with the results interpretation. Largely, this only requires that the species to which the GMO can belong are identified.

If the product subject to testing can contain material (DNA) from more than a single species, it is strongly recommended to consider the inclusion of species identification screenings. Indeed, the list of species for which one or more GMOs have been developed, field-trialed or commercialized is still quite limited (cf. BCH, 2011; CERA, 2011; GMO Compass, 2011).

Experience has shown that for many products containing soybean and/or maize, presence of some GMO derived material (DNA) is very likely. This is particularly true for feeds. For these products it is therefore often more relevant to consider the legal status of present GMO(s) and whether or not the quantity exceeds relevant threshold(s) such as a threshold for labeling of GM products.

Examples of how the matrix approach has been implemented into rather complex analytical tools include the DualChip® GMO developed by Eppendorf Array Technologies (Hamels et al., 2009; Leimanis et al., 2008), the SNPlex assay developed by Chaouachi et al. (2008) and the GMOseek project (Querci et al., 2010).

National networks of GMO laboratories in e.g. Belgium (Van den Bulcke et al., 2010), Germany (Waiblinger et al., 2010) and Japan (Mano et al., 2009) have established formal national guidelines to GMO testing. The guidelines usually apply the matrix approach and typically start with screening for particular species and single elements and constructs. In Germany, this has been more extensively elaborated than in any other country. At present five element- or construct-specific modules have been validated through collaborative

Species	GMO	Legal status	Analytical module - specificity										ctp2-cp4epsps Construct G	P35S-pat Construct H
			CaMV P35S Promoter A	FMV P35S Promoter B	aad Gene C	bar Gene D	npII Gene E	pat Gene F	T-nos Terminator E	T-E9 Terminator F				
Canola/rapeseed	Falcon GS40/90	Un-authorized	+	-	-	-	-	+	-	-	-	+		
	GT73	Authorized	-	+	-	-	-	-	-	+	+	-		
	Topas 19/2	Un-authorized	+	-	-	-	+	+	-	-	-	+		
	MS1, RF1, RF2	Un-authorized	-	-	-	+	+	-	+	-	-	-		
	MS8, RF3	Authorized	-	-	-	+	-	-	+	-	-	-		
	OXY235	Un-authorized	+	-	-	-	-	-	+	-	-	-		
Cotton	281-24-236	Un-authorized	-	-	-	-	-	+	-	-	-	-		
	3006-210-23	Un-authorized	-	-	-	-	-	+	-	-	-	-		
	GH8614	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	LL25	Authorized	+	-	-	+	-	-	+	-	-	-		
	MON531	Authorized	+	-	+	-	+	-	-	-	-	-		
	MON1445	Authorized	+	+	+	-	+	-	+	+	+	-		
	MON15985	Authorized	+	-	+	-	+	-	+	-	+	-		
	MON88913	Un-authorized	+	+	-	-	-	-	-	+	+	-		
Linseed/flax	FP967	Un-authorized	-	-	+	-	+	-	+	-	-			
Maize/corn	Bt11	Authorized	+	-	-	-	+	+	+	-	-	+		
	Bt176	Un-authorized	+	-	-	+	-	-	-	-	-	-		
	DAS-40278-9	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	GA21	Authorized	-	-	-	-	-	-	+	-	-	-		
	LY038	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	MIR604	Un-authorized	-	-	-	-	-	-	+	-	-	-		
	MON810	Authorized	+	-	-	-	-	-	-	-	-	-		
	MON863	Authorized	+	-	-	-	+	-	+	-	-	-		
	MON87460	Un-authorized	+	-	-	-	+	-	+	-	-	-		
	MON88017	Authorized	+	-	-	-	-	-	-	-	+	-		
Papaya	55-1, 63-1	Un-authorized	+	-	-	-	+	-	+	-	-	-		
	X17-2	Un-authorized	+	-	-	-	+	-	-	-	-	-		
Potato	EH92-527-1	Authorized	-	-	-	-	+	-	+	-	-	-		
	AM-04-1020	Un-authorized	-	-	-	-	-	-	+	-	-	-		
	AV43-6-G7	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	RBMT21 (New leaf)	Un-authorized	-	+	-	-	+	-	+	+	-	-		
	RBMT22 (New leaf)	Un-authorized	-	+	+	-	+	-	+	+	+	-		
Rice	Bt63	Un-authorized	-	-	-	-	-	-	+	-	-	-		
	KeFeng 6	Un-authorized	+	-	-	-	-	-	+	-	-	-		
	KMD1	Un-authorized	-	-	-	-	-	-	+	-	-	-		
	LL62	Un-authorized	+	-	-	+	-	-	-	-	-	-		
	LL601	Un-authorized	+	-	-	+	-	-	-	-	-	-		
Soybean	305423	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	356043	Un-authorized	+	-	-	-	-	-	-	-	-	-		
	A2704-12	Authorized	+	-	-	-	-	+	-	-	-	+		
	A5547-127	Un-authorized	+	-	-	-	-	+	-	-	-	+		
	DAS-68416-4	Un-authorized	-	-	-	-	-	+	-	-	-	-		
	GTS40-3-2	Authorized	+	-	-	-	-	-	+	-	-	-		
	MON87701	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	MON87705	Un-authorized	-	+	-	-	-	-	-	+	+	-		
	MON87708	Un-authorized	-	-	-	-	-	-	-	+	-	-		
	MON87769	Un-authorized	-	-	-	-	-	-	-	+	-	-		
Sugarbeet	GTS877	Un-authorized	+	+	-	-	-	-	-	+	+	-		
	H7-1	Authorized	-	+	-	-	-	-	-	+	+	-		
	T120-7	Un-authorized	+	-	-	-	+	+	-	-	-	+		
Tomato	35.1 N	Un-authorized	-	-	-	-	+	-	+	-	-	-		
	1345-4 (Endless summer)	Un-authorized	+	-	-	-	+	-	+	-	-	-		
	5345	Un-authorized	+	-	+	-	+	-	+	-	-	-		
	8338	Un-authorized	+	+	-	-	+	-	+	+	-	-		
	CGN-89564-2 (FlavrSavr)	Un-authorized	+	-	-	-	-	-	-	-	-	-		
			CaMV P35S Promoter A	FMV P35S Promoter B	aad Gene C	bar Gene D	npII Gene E	pat Gene F	3'-nos Terminator E	3'-E9 Terminator F	ctp2-cp4epsps Construct G	P35S-pat Construct H		

Fig. 4. Example of a matrix with ten analytical modules and data tabulated from ten plant species. NB: the matrix shown is not complete with respect to existing GMOs on the global market. It is only meant to illustrate the application of the matrix approach with examples in the following part of this review. GMOs that are not detectable with any of the ten analytical modules are indicated in yellow. For these, detection can only be achieved with modules not included in the shown matrix, e.g. event specific modules. Presence of the target (detectable with the analytical module) is indicated with a +, while absence (not detectable) is indicated with a -. The authorization status for any GMO will depend on the jurisdiction. The status indicated here is only intended for illustration purposes and is not intended to reflect the actual authorization status in any jurisdiction.

trials (see Waiblinger et al., 2010 and references therein). The performance has been verified against reference materials of ten rapeseed events, nine cotton events, one linseed (flax) event, 19 maize events, one papaya event, two potato events, five rice events, seven soybean events and three sugar beet events. Out of nearly 100 GMOs belonging to the listed species + tomato, only 12 are reported not to contain any of the five screening targets (see the official matrix online: http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/screening_tabelle_gvoNachweis.xls?__blob=publicationFile). Notably, however, the theoretical presence needs to be experimentally verified for approximately 30 of the nearly 100 listed GMOs. This task is mainly depending on the availability of reliable reference material. For some of the GMOs, there will probably never be certified material available, but if DNA sequence data are available then synthetic DNA fragments can be an alternative.

To facilitate result interpretation in the analytical laboratories the electronic versions of the matrixes of the Belgian, German and Japanese GMO testing systems have been supplemented with simple software such as scroll down option menus in Excel. This software allows the analyst to automatically obtain a list of the GMOs that can be

present in the sample, given the observed presence/absence of analytical targets. Related decision support software tools have been developed in various, mainly EU-funded, projects. Some of these tools allow the user to input additional information on (new) analytical modules and GMOs. Examples include the Co-Extra decision support system (Bohanec et al., 2013), the GMOTrack (Novak et al., 2009) and the GMOseek (unpublished data).

6. Qualitative screening

Initially, when samples are analyzed, the most commonly applied PCR modules are element specific screening modules. The resulting presence/absence pattern is compared to the reference pattern of the matrix, and it immediately becomes possible to part out the GMOs that are not detectable at the LOD_{pract}. This will normally reduce the list of GMOs that can be present in the sample significantly. Furthermore, it will often help the analyst to identify PCR modules that will provide the most information if applied successively. This process can go through several rounds of screenings, but in most cases it is not necessary to apply more than a few screening

modules/rounds to narrow the list down to a level where event specific modules can be applied reasonably for confirmation. If a positive screening result is obtained, but no corresponding GMO is identified successively, there is usually the possibility to perform additional analyses to verify the result, e.g. the use of tail-PCR (Liu et al., 1995), anchor PCR (Theuns et al., 2002), cloning and DNA sequencing. This is particularly relevant in case of samples containing ISK-class 3 GMOs. Such ad hoc analytical work is not commonly performed by routine laboratories, and due to potentially high costs and uncertain outcome it is not evident if and eventually when such work is justifiable.

7. Exploiting quantitative information

Many screening modules are real-time PCR modules and therefore potentially quantitative by nature. But they are usually less reliable than event specific modules when it comes to quantification. Two significant uncertainties are the cause of this reduced reliability. Allelic variation can affect the amplification efficiency of a target (Ghedira et al., 2009; Papazova et al., 2010), as also exemplified with a CaMV P35S specific screening module discussed by Morisset et al. (2009). In Bt11 maize a CaMV P35S-*pat* construct can have significantly reduced detectability compared to e.g. TC1507 maize due to the length of the amplicon (Waiblinger et al., 2010). In extreme cases, such as for the CaMV P35S-*pat* construct in Bt11 in highly processed maize samples, or with unfortunate nucleotide substitutions, the target can even become undetectable.

The number of target copies in different GMOs can also vary, both between homo-, hemi- and heterozygous GM plant tissues (Cankar et al., 2008; Holst-Jensen et al., 2006 and references therein) and with respect to number of inserted copies integrated during transformation. The two soybean events A5547-127 and GU262 for example contain one and two copies, respectively, of a construct in which the CaMV P35S and *pat* gene is present (CERA, 2011). Other examples can be found in Cankar et al. (2008).

The reliability of quantitative estimates is strongly influenced by sampling and measurement uncertainty. Sampling particularly affects the LOD_{pract} and LOQ_{pract}. Non-representative sampling will result in significantly inferior values (higher LOD/LOQ) than estimated from PCR data (see also Sections 2.2.1 and 10.3). Details on the sampling are not always known by the stakeholder who therefore inadvertently may believe that the LOD/LOQ is better than it is. The interested reader is referred to Sustar-Vozlic et al. (2010) and references therein for more comprehensive discussion of GMO sampling issues. Guidelines to estimation of measurement uncertainty in GMO testing are published by Zel et al. (2007) and Trapmann et al. (2009). The measurement uncertainty must always be taken into consideration when interpreting quantitative data.

Despite a number of pitfalls, it can be useful to obtain quantitative data in connection with screenings. If the absolute quantity of a screening marker is at or below the LOD_{abs}, then any present GMO containing this marker has a significant risk of producing a false negative test result with an event specific module. A very high concentration of a specific screening marker on the other hand is a clear indication of a very high GMO concentration, possibly high enough to render more specific quantitation (but not identification) superfluous. Cankar et al. (2008) proposed to use such discrepancies in the observed quantities between screening and event specific data in a “differential quantitative PCR approach” to identify samples with possible presence of un-authorized GMOs. The idea is to use statistics and assess the power of the statistical test to determine if the observed quantity of a screening marker exceeds what can be explained by the observed cumulative quantities of authorized events and non-GM donors. If this is the case then the statistically significant excess quantity of the screening marker is a strong indication of the presence of un-authorized GMO.

Many GMO testing laboratories apply the same principles in their day-to-day decisions on further confirmatory testing after an initial screening. For example, if a sample of maize is found to contain CaMV P35S and T-*nos* in similar quantities, but also contains DNA from soybean, then a confirmatory quantitative test for presence of the GM soybean event GTS 40-3-2 (Roundup Ready) may be sufficient. GTS 40-3-2 is at present the single GMO with the widest global distribution and acreage. If the test reveals presence of GTS 40-3-2 in a quantity similar to that of CaMV P35S and T-*nos*, then it may be justifiable not to perform additional event specific tests based on the CaMV P35S and T-*nos* results. However, presence of other GMOs, including un-authorized events cannot be completely excluded based on this more simplified application of the differential quantitative PCR principle. The reliability of the differential quantitative approach is currently under study, using inter laboratory proficiency testing scheme data.

8. Exploiting traceability data and publicly available information

Data on the origin of a sample can be very useful. Some GMOs are only cultivated in one or a few countries, e.g. Bt63, KeFeng-6 and KMD1 rice in China (Reiting et al., 2010), while others are cultivated on a large scale worldwide, e.g. GTS 40-3-2 soybean (James, 2011). The cost-efficiency of a testing scheme can be improved if this type of information is taken into consideration. Then the testing can typically focus on the GMOs most likely to be present in a sample. Various decision support tools such as the Co-Extra DSS (Bohanec et al., 2013), the GMOtrack (Novak et al., 2009) and the GMOseek (unpublished data) are designed to do this. But, available information can also be misleading, in which case reliance on stated origin can result in failure to detect present GMO. Examples will be presented in Section 10.

Ruttink et al. (2010b) demonstrated the potential of exploiting information-knowledge to detect a GMO placed on the market without the necessary authorization. They proposed a distinction between the traditional analyte centered approach, such as the matrix approach, and an information-knowledge based approach. The starting point was information about new biotech products under development and in the pipeline for marketing. Ruttink et al. (2010b) mainly retrieved information from the internet using the search engine Google. They used keyword based searches referring to company and product name and more technical terms such as the short name of the trait gene and the Latin binomial of the donor and recipient species. This allowed Ruttink et al. (2010b) to retrieve enough information to design a test method and locate samples that could be tested. Detection of the targeted un-authorized GMO in these samples was a clear proof of concept. Since no specific detection method for this GMO was available a priori the analyte centered approach would have failed in this case. Despite the apparent success there is yet no example of the application of this approach to other cases.

9. Reference materials

Reference materials in GMO analysis serve as positive and negative controls and as calibrants for quantitative analyses. For many un-authorized GMOs there is no certified reference material (CRM) available. In some cases it is possible to prepare a synthetic oligonucleotide or cloned fragment with the same DNA sequence as the intended target. The commutability of such control samples to the corresponding GMO is, unfortunately, inferior to CRMs. Proficiency testing samples is another commonly used source of positive controls. Accreditation bodies have very strict requirements, among others for the quality of reference or control materials. For many un-authorized GMOs it will not be possible to meet these requirements, and thus it will not be possible to apply the detection methods for these GMOs

under full ISO 17025 compliance. Detection of un-authorized GMO is therefore only exceptionally done under ISO 17025 accreditation.

The availability of reference materials will vary, as particular batches can be sold out or retracted. The German network of GMO laboratories has prepared a comprehensive list of globally available reference materials for GMOs. This list is published on the internet (http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/nachweis_kontrollen/referenzmaterialien.pdf?__blob=publicationFile) and is updated several times per year. The list includes all types of reference materials.

Reference materials and other control samples are usually only characterized with respect to presence/absence of a limited set of targets. This means that the reference materials and control samples sometimes also contain non-declared targets. Non-declared targets can for example be traces of another GMO than the one for which the sample is certified. It is important to appreciate this when applying the reference materials and control samples for example during specificity testing of analytical modules and when results are interpreted in the screening phase before choosing the event-specific modules.

There are pros and cons with every type of reference material. The target sequence motif of a particular detection module can be amplified e.g. using PCR technology or it can be cloned separately or as part of larger constructions and propagated in plasmids in bacteria. Such reference materials, once developed, can be easily distributed, purified and produced in large quantities. As such they may fit well with the modular approach described in Section 2. Genomic DNA purified from a specific GMO is perhaps more representative of the GMO and materials subject to analysis, mainly because of its complexity. This includes potentially interfering DNA sequence motifs and need for removal of proteins and other potentially inhibitory substances prior to the analysis. It is sometimes reported that cloned DNA and genomic DNA can exhibit different amplification efficiencies. Other reports indicate that this is perhaps only due to incomplete purification or degradation of the genomic DNA. Several of the available CRMs are kernels or flours of GMOs, blended or not with non-GM kernels or flours. The certification is a guarantee of commutability. However, CRMs may be less suited for the PCR analysis itself due to possible presence of non-declared targets and difficulties with the DNA purification and quality. For more comprehensive discussions on the performance of different types of reference materials we refer e.g. to Burns et al. (2006) and Charels et al. (2007).

10. Analytical detection of un-authorized GMOs in practice

10.1. Direct detection of a registered, un-authorized GMO

The simplest scenario is one where the presence of a specific un-authorized GMO is suspected in particular samples. For example, in 2006, after the announcement of its unintended release by Bayer CropScience the observed presence of the un-authorized rice event LL601 in U.S. shipments to Europe resulted in the launch of emergency measures (European Commission, 2006a). The official control laboratories in the EU and Norway then performed analyses directly targeting this single event in all rice lots originating from the U.S.A. The matrix approach and broad screening were not applied in this case. Instead, the laboratories used a construct-specific PCR module, see EURL-GMFF (2006a, 2006b) for more details. But, relying too much on the stated origin of a product can result in failure to detect present GMO.

Until today, the only GM rice events detected in routine in rice from the U.S.A. are LL62 and LL601. In 2010, a sample stated to be “wild rice from U.S.A.” was received for analysis by the Norwegian Veterinary Institute. Instead of analyzing directly for the LL rice events, the sample was subjected to broader screening analysis. The results indicated possible presence of another un-authorized rice

event, viz. Bt63. But Bt63 GM rice was to date only traced in Chinese rice and had never been found in rice originating from outside of Asia. Further analyses confirmed that the sample contained Bt63 (RASFF reference no. 2010.0853), and it was later made clear that the wild rice from U.S.A. had been mixed with rice from Asia after arrival to Europe. So the true origin of the Bt63 rice in the sample was concluded to be Asia, not the U.S.A. This Bt63 rice would never have been detected if the sample had been analyzed based on traceability documents considering the claimed origin of the material solely. A similar case was reported from Slovenia on a shipment of linseed (flax) from China via Germany (RASFF reference no. 2009.1640). The GMO in question (FP967) had previously only been reported from Canada.

10.2. Limited screening-based GMO testing of products consisting of a single or a low number of ingredients

Prior to analysis, it is necessary to clarify if a particular screening-based PCR approach covers all authorized GMOs registered for a specific set of ingredients/species present in the sample. Sometimes, one or more GMOs of a species contain none of the common screening elements (cf. Fig. 4, events highlighted in yellow). In the latter situation, it will be necessary to apply a broader range of PCR tests to determine the presence/absence status of the(se) GMO(s). If legally only official ingredients have to be considered, then non-ingredient species that are also present in the sample are formally irrelevant. But this does not mean that they cannot interfere with the analysis and confuse the results interpretation (Berben et al., 2009). The analysis of two example products presumably containing a single ingredient (= species) is described in the following. Four scenarios are described in Fig. 5. In this example the ingredient species is rice. Because in our example matrix there is no authorized GM rice event, the question to be answered is: does the sample contain an un-authorized GMO?

The LOD is always a critical matter. The LOD_{abs} is usually around 5–10 copies of the target. At near LOD concentrations there is always a significant risk of false negative results for individual tests. This of course must be taken into consideration when analytical results are interpreted. If two screening targets are present in a GMO but at different insert copy numbers, e.g. one and four, the relative LOD for these will differ four-fold for a DNA solution obtained solely from that GMO. Unfortunately, the analyst will often not have detailed information on the copy number inserted for each target in each GMO. Thus, the pragmatic approach will be to consider not only the observed presence/absence pattern but also the approximate absolute concentration of detected targets. The latter can be extrapolated from standard curves, of course bearing in mind the possibility that the target present in the GMO may exhibit slightly divergent PCR performance from the target present in the standards. It is reasonable to assume (based on publicly available data) that the vast majority of GMOs exhibit copy number ratios of insert targets in the range of 1:1 and 1:5. Thus, if one assumes a LOD_{abs} of 10 copies, then observed presence of approx. 50 copies of a target known to be present in a particular GMO would imply that it is unlikely that the particular GMO is present if another target known to be present in that GMO is not detectable in the sample. Scenarios 2 and 3 described with Fig. 5 are examples where (semi-)quantitative information could have relevance for correct results interpretation.

Two scenarios are described in Fig. 6. In this example, the ingredient species is soybean. Because there are both authorized and un-authorized GM soybean events listed in our example matrix, there are at least two questions that may need to be answered, depending on the jurisdiction: 1) Does the sample contain any un-authorized GMO (soybean)? 2) Does the sample contain so much authorized GM-soybean that it has to be labeled (in the EU > 0.9%)? Current practice among GMO testing laboratories varies. Some design their testing strategy to focus initially on the first question, usually limited to GMO








Species	GMO	Legal status	Analytical module - specificity										ctp2-cp4epsps Construct G	P35S-pat Construct H
			CaMV P35S Promoter A	FMV P35S Promoter B	aad Gene C	bar Gene D	nptII Gene E	pat Gene F	T-nos Terminator E	T-E9 Terminator F				
Rice	 Bt63	Un-authorized	-	-	-	-	-	-	+	-	-	-		
	 KeFeng 6	Un-authorized	+	-	-	-	-	-	+	-	-	-		
	 KMD1	Un-authorized	+	-	-	-	-	-	+	-	-	-		
	 LL62	Un-authorized	+	-	-	+	-	-	-	-	-	-		
	 LL601	Un-authorized	+	-	-	+	-	-	-	-	-	-		
Soybean	 305423	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	356043	Un-authorized	+	-	-	-	-	-	-	-	-	-		
	A2704-12	Authorized	+	-	-	-	-	+	-	-	-	+		
	A5547-127	Un-authorized	+	-	-	-	-	+	-	-	-	+		
	DAS-68416-4	Un-authorized	-	-	-	-	-	+	-	-	-	-		
	 GTS40-3-2	Authorized	+	-	-	-	-	-	+	-	-	-		
	MON87701	Un-authorized	-	-	-	-	-	-	-	-	-	-		
	MON87705	Un-authorized	-	+	-	-	-	-	-	+	+	-		
	MON87708	Un-authorized	-	-	-	-	-	-	-	+	-	-		
	MON87769	Un-authorized	-	-	-	-	-	-	-	+	-	-		
	MON89788	Authorized	-	+	-	-	-	-	-	+	+	-		

Fig. 5. A putative single ingredient product (rice) is subjected to quantitative analysis using a real-time quantitative PCR module targeting a single copy housekeeping rice gene and screening with only two analytical modules: CaMV P35S and T-nos. The quantity of the single copy housekeeping rice gene indicates that the practical limit of detection for any GM-associated target is 0.05%. All GM rice events are un-authorized. Scenario 1: Both screening modules show negative test results, and the interpretation is that GMO is not detectable at an LOD of 0.05%. Scenario 2: The CaMV P35S is detected, while the T-nos is not. The interpretation is that the sample may contain LL62 and/or LL601 rice (red arrows). Confirmatory analysis can either be performed directly using two event specific modules, or screening with the single module targeting the *bar* gene. A negative test result with the *bar* gene indicates that the source of the CaMV P35S is not one of the listed rice GMOs, while a positive test result will require final confirmatory testing with LL rice specific modules. Scenario 3: The T-nos is detected while the CaMV P35S is not. The interpretation is that the sample may contain Bt63 rice (blue arrow), and confirmatory analysis is then performed using a Bt63 specific module. Scenario 4: Both screening modules are detected, and the interpretation is that the sample may contain KeFeng-6 or KMD 1 rice (green arrows) or a mix of two or more of the GM rice events. This can only be confirmed using modules specific to one or more of the GM rice events. However, when GM screening targets are detected, there is also another possible explanation; – the presence of a non-declared impurity of GM-origin such as a GM soybean. Interpretation of the analytical results therefore should take into consideration also the other species represented in the matrix. In scenario 4 the presence of both the CaMV P35S and the T-nos is compatible with the presence of the authorized GM soybean event GTS 40-3-2 (black arrow).

of the ingredient species, but exceptionally also taking into consideration other species. Other laboratories only focus on the first question if there is reason to suspect that un-authorized GM soybean is present. Suspicion can for example be based on presence/absence patterns of screening targets that do not match the patterns predicted by the matrix if only authorized GM soybean is present in the sample.

10.3. Complex screening-based GMO testing of products containing multiple ingredients and/or where DNA quantity and/or integrity are significantly affected by processing

Samples composed of several ingredients and/or with ingredients that have been subjected to strong DNA-degrading processes are often complicated to test. DNA degradation significantly reduces the detectability of GMOs because fewer intact copies of the target sequence will be present per mass (volume) unit of the sample material

(Gryson, 2010). Mixing of ingredients reduces the relative fraction that is derived from a specific ingredient, and thus also the relative mass fraction of the total DNA derived from that ingredient in the sample (Gryson, 2010). The LOD_{pract} for any GMO is a function of the absolute quantity of DNA of the ingredient that is available for PCR analysis (Holst-Jensen et al., 2003). The LOD_{pract} is therefore affected by processing and mixing of ingredients. Notably, the DNA must be of PCR-grade quality, i.e. structurally intact and virtually free of inhibitors of the PCR. DNA can also act as an inhibitor in case the total mass exceeds a certain concentration (e.g. >200 ng/μl for maize DNA samples).

So, the first problem is the rather low absolute quantity of target DNA that can be included in a PCR. The second problem is the reduced ability to discriminate between GMOs because of the possible larger number of candidates that can explain the profile of detected screening targets. The same screening PCR target can indeed be derived




Species	GMO	Legal status	Analytical module - specificity										ctp2-cp4epsps Construct G	P35S-pat Construct H
			CaMV P35S Promoter A	FMV P35S Promoter B	aad Gene C	bar Gene D	nptII Gene E	pat Gene F	T-nos Terminator E	T-E9 Terminator F				
Soybean	 305423	Un-authorized	-	-	-	-	-	-	-	-	-	-	-	
	 356043	Un-authorized	+	-	-	-	-	-	-	-	-	-	-	
	A2704-12	Authorized	+	-	-	-	-	+	-	-	-	-	+	
	A5547-127	Un-authorized	+	-	-	-	-	+	-	-	-	-	+	
	DAS-68416-4	Un-authorized	-	-	-	-	-	+	-	-	-	-	-	
	GTS40-3-2	Authorized	+	-	-	-	-	-	+	-	-	-	-	
	 MON87701	Un-authorized	-	-	-	-	-	-	-	-	-	-	-	
	MON87705	Un-authorized	-	+	-	-	-	-	-	+	-	+	-	
	MON87708	Un-authorized	-	-	-	-	-	-	-	+	-	-	-	
	MON87769	Un-authorized	-	-	-	-	-	-	-	+	-	-	-	
MON89788	Authorized	-	+	-	-	-	-	-	+	-	+	-		

Fig. 6. A sample consisting of soybeans is tested. In this case, presence of GMOs of other species is irrelevant, unless it could affect results in a way that would lead to (legal) action. The limit of detection is a function of the number of soybeans from which DNA is extracted. In this case approx. 10⁴ soybeans are ground to flour and DNA is extracted from this flour. The application of a soybean specific real-time PCR module confirms that the limit of detection is better than 0.1% for a single copy target. The first step is testing with five analytical modules: screening with the CaMV P35S, *pat* and T-E9 specific modules, plus two event specific modules targeting 305423 and MON87701 (red arrows), respectively. The latter modules are needed because these two events (highlighted in yellow) cannot be detected with any of the available screening modules listed in the matrix. Scenario 5: All analytical modules show negative test results, and the interpretation is that GMO is not detectable in the sample at an LOD better than 0.1%. Scenario 6: The CaMV P35S is detected, but the other screening targets are not. The interpretation is that the sample may contain the authorized GM-soybean event GTS 40-3-2 and/or the un-authorized soybean event 356043. It is only necessary to test for GTS 40-3-2 if the quantity can exceed a relevant (e.g. labeling) threshold. If CaMV P35S is detected with a quantitative real-time PCR module, then the quantity of this target can provide sufficient information to determine if testing for GTS 40-3-2 is necessary or not. Testing for 356043 soybean (blue arrow) has to be done using an event specific module. Several additional scenarios can be imagined but are not treated here. Some of these will in part be described and discussed in the example presented in Fig. 8.

from GMOs that belong to a different ingredient/species (cf. Fig. 4). Furthermore, presence of for example three screening elements in a sample composed of three species can be due to presence of one or several GMOs containing none, one, two or even all three elements in each of the three species. A similar problem is encountered when stacked GMOs are present in a sample. This means that particular screening results can be difficult to interpret.

The first level of information required for correct interpretation and further design of the analytical procedure concerns the presence and quantity of DNA of relevant species in the sample. The LOD_{abs} is typically in the range of 5 to 10 target copies for GMO screening modules. Thus, if the number of haploid soybean genomes (haploid genome equivalents; HGE) in the extracted DNA is estimated to 5×10^3 per PCR, then the LOD_{pract} for soybean GMO would be around 0.1% ($= 5 \text{ target HGE} / 5 \times 10^3 \text{ species HGE}$). But if for example the quantity of DNA of another species in the same sample is estimated to be as low as 25 haploid genomes, then the LOD_{pract} for the same screening target in that species is theoretically close to 20% ($= 5 \text{ target HGE} / 25 \text{ species HGE}$).

As is the case in simple GMO screening approaches, here it is also necessary to clarify whether a screening PCR approach is sufficient to detect all GMOs belonging to the species present, or whether there are GMOs of any of the species that do not contain any of the screening elements (cf. the matrix). In the latter situation, it will be necessary to apply more specific PCR tests to determine the presence/absence status of the (se) GMO(s).

After conducting the screening, the results can be interpreted by matching with the patterns described in the matrix. In the following, six scenarios are described, using two examples of products. In Fig. 7, the sample is maize/corn gluten feed. The presence of soybean

derivatives in the maize gluten is likely, and therefore has to be taken into consideration when the results are interpreted. But soybean is a “botanical impurity” and not an official ingredient, and therefore exempt from labeling for example in the EU (Berben et al., 2009). Whether or not presence of un-authorized GMO in a non-ingredient is illegal depends on the jurisdiction. In the EU such presence is illegal (European Commission, 2003a). In Fig. 8, the sample is a feed produced from multiple ingredients and likely to contain DNA also from non-declared (plant) species.

11. Alternative and advanced technologies for routine and ad hoc application

The technological advances in molecular biology gradually increase the number of GMOs whose inserts do not contain any of the genetic elements inserted into the currently commercial GMOs. Consequently, the risk that a GMO is present in a sample but remains effectively undetectable with the currently applied detection modules is real. A robust, cost-efficient solution to this new challenge is not evident, although a few alternative and advanced technologies have been proposed along with proof of concept.

These technologies are often more expensive and require extensive, specialized manpower and time. They can be difficult to standardize and validate through collaborative trials. Therefore, for many of these technologies the application in routine testing as such is unlikely. However, there is some potential for simplification and ad hoc design, e.g. adapted to particular species. Furthermore, if samples subjected to analyses with one of these technologies are taken on the basis of specific suspicion (cf. Ruttink et al., 2010b) then some kind of information that may give clues to the nature of

Species	GMO	Legal status	Analytical module - specificity									
			CaMV P35S Promoter A	FMV P35S Promoter B	aad Gene C	bar Gene D	nptII Gene E	pat Gene F	T-nos Terminator E	T-E9 Terminator F	ctp2-cp4epsps Construct G	P35S-pat Construct H
Maize/corn	Bt11	Authorized	+	-	-	-	-	+	-	-	-	-
	Bt176	Un-authorized	+	-	-	+	-	+	-	-	-	-
	DAS-40278-9	Un-authorized	-	-	-	-	-	-	-	-	-	-
	GA21	Authorized	-	-	-	-	-	-	+	-	-	-
	LY038	Un-authorized	-	-	-	-	-	-	-	-	-	-
	MIR604	Un-authorized	-	-	-	-	-	-	+	-	-	-
	MON810	Authorized	+	-	-	-	-	-	-	-	-	-
	MON863	Authorized	+	-	-	-	+	-	+	-	-	-
	MON87460	Un-authorized	+	-	-	-	+	-	+	-	-	-
	MON88017	Authorized	+	-	-	-	-	-	+	-	+	-
	MON89034	Authorized	+	+	-	-	-	-	+	-	-	-
Soybean	305423	Un-authorized	-	-	-	-	-	-	-	-	-	-
	356043	Un-authorized	+	-	-	-	-	-	-	-	-	-
	A2704-12	Authorized	+	-	-	-	-	+	-	-	-	+
	A5547-127	Un-authorized	+	-	-	-	-	+	-	-	-	+
	DAS-68416-4	Un-authorized	-	-	-	-	-	+	-	-	-	-
	GTS40-3-2	Authorized	+	-	-	-	-	-	+	-	-	-
	MON87701	Un-authorized	-	-	-	-	-	-	-	-	-	-
	MON87705	Un-authorized	-	+	-	-	-	-	-	+	+	-
	MON87708	Un-authorized	-	-	-	-	-	-	-	+	-	-
	MON87769	Un-authorized	-	-	-	-	-	-	-	+	-	-
	MON89788	Authorized	-	+	-	-	-	-	-	+	+	-

Fig. 7. A sample consisting of maize gluten feed is analyzed. The following only describes selected examples of the possible scenarios. Scenario 7: The quantity of maize derived DNA determined with a real-time quantitative PCR module is too low to offer acceptable limits of detection (LOD) and quantitation (LOQ). Negative test results are therefore of limited use. Scenarios 8–10: The quantity of maize derived DNA indicates a LOD_{pract} of 0.1% and a LOQ_{pract} of 0.5% for a single copy target. The sample is first subjected to screening with the three modules specific to CaMV P35S, *nptII* and T-nos and analysis with two event specific modules targeting DAS-40278-9 and LY038 (red arrows), respectively. The latter modules are needed because these two events (and others also highlighted in yellow) cannot be detected with any of the available screening modules listed in the matrix. The CaMV P35S and T-nos tests are quantitative. Scenario 8: The only modules yielding positive test results are the CaMV P35S ($0.5\% \pm 0.3\%$) and the T-nos ($0.7\% \pm 0.4\%$). The interpretation is that if the sample contains authorized GM-maize, it is not required to label (at a threshold of 0.9%). It is possible that the sample contains the un-authorized GM-maize events Bt176 and/or MIR604 (blue arrows). Testing with event specific modules targeting these events is therefore required. Scenario 9: The results are the same as in scenario 8, except that the *nptII* module also yields a positive test result < LOQ. This target is present in two GM-maize events; – the authorized MON863 and the un-authorized MON87460 (green arrow). An event specific test targeting the latter is therefore required. Scenario 10: The results are the same as in scenario 8, except that the quantities of CaMV P35S ($2.9\% \pm 1.5\%$) and T-nos ($2.4\% \pm 1.2\%$) are higher. The interpretation is that the sample may have to be labeled. Further testing is required. The single most frequently detected GMO in the agricultural supply chain is the soybean event GTS 40-3-2, and this event contains both the CaMV P35S and the T-nos. Testing for presence of soybean and this event indicates the presence of a small but significant amount of soybean of which nearly 100% is GTS 40-3-2. The analytical laboratory translates the results into target copy numbers and applies a simplified form of differential quantitative PCR. The conclusion is that GTS 40-3-2 is the likely source of more than 50% of the total quantity of these screening targets. Given the measurement or statistical uncertainty, it is concluded that it is impossible to determine with necessary certainty whether the quantity of authorized GM-maize events exceeds the threshold for labeling (0.9%). Thus, the only basis for action is if an un-authorized GM-maize event is detected. The same tests for unauthorized GMOs as applied in scenario 8 are therefore applied.

Species	GMO	Legal status	Analytical module - specificity										ctp2-cp4epsps Construct G	P35S-pat Construct H
			CaMV P35S Promoter A	FMV P35S Promoter B	aad Gene C	bar Gene D	nptII Gene E	pat Gene F	T-nos Terminator E	T-E9 Terminator F				
Canola/rapeseed	Falcon GS40/90	Un-authorized	+	-	-	-	-	+	-	-	-	-	-	+
	GT73	Authorized	-	+	-	-	-	-	-	+	-	+	-	-
	Topas 19/2	Un-authorized	+	-	-	-	+	+	-	-	-	-	-	+
	MS1, RF1, RF2	Un-authorized	-	-	-	+	+	-	+	-	-	-	-	-
	MS8, RF3	Authorized	-	-	-	+	-	-	+	-	-	-	-	-
	OXY235	Un-authorized	+	-	-	-	-	-	+	-	-	-	-	-
Maize/corn	Bt11	Authorized	+	-	-	-	-	+	+	-	-	-	-	+
	Bt176	Un-authorized	+	-	-	+	-	-	-	-	-	-	-	-
	DAS-40278-9	Un-authorized	-	-	-	-	-	-	-	-	-	-	-	-
	GA21	Authorized	-	-	-	-	-	-	+	-	-	-	-	-
	LY038	Un-authorized	-	-	-	-	-	-	-	-	-	-	-	-
	MIR604	Un-authorized	-	-	-	-	-	-	+	-	-	-	-	-
	MON810	Authorized	+	-	-	-	-	-	-	-	-	-	-	-
	MON863	Authorized	+	-	-	-	+	-	+	-	-	-	-	-
	MON87460	Un-authorized	+	-	-	-	+	-	+	-	-	-	-	-
	MON88017	Authorized	+	-	-	-	-	-	+	-	-	+	-	-
	MON89034	Authorized	+	+	-	-	-	-	+	-	-	-	-	-
Soybean	305423	Un-authorized	-	-	-	-	-	-	-	-	-	-	-	-
	356043	Un-authorized	+	-	-	-	-	-	-	-	-	-	-	-
	A2704-12	Authorized	+	-	-	-	-	+	-	-	-	-	-	+
	A5547-127	Un-authorized	+	-	-	-	-	+	-	-	-	-	-	+
	DAS-68416-4	Un-authorized	-	-	-	-	-	+	-	-	-	-	-	-
	GTS40-3-2	Authorized	+	-	-	-	-	-	+	-	-	-	-	-
	MON87701	Un-authorized	-	-	-	-	-	-	-	-	-	-	-	-
	MON87705	Un-authorized	-	+	-	-	-	-	-	+	-	+	-	-
	MON87708	Un-authorized	-	-	-	-	-	-	-	+	-	-	-	-
	MON87769	Un-authorized	-	-	-	-	-	-	-	+	-	-	-	-
	MON89788	Authorized	-	+	-	-	-	-	-	+	-	+	-	-

Fig. 8. Testing for presence/absence of un-authorized GMO in a feed sample made from multiple ingredients. Initially, tests are performed to identify the species from which DNA is present in the sample. The following only describes selected examples of the many possible scenarios. DNA from canola/rapeseed, maize/corn and soybean are detected. Screening is performed only with three modules targeting the *nptII* gene, the *pat* gene and the T-E9, respectively. In this case, screening is not performed with the modules targeting the CaMV P35S and T-nos because it is predicted that both will yield positive results and that the information associated with such a test result will be of little use with respect to detection of un-authorized GMO. For laboratories wishing to test for compliance with quantitative labeling requirements, however, such data are potentially beneficial. Scenario 11: The *nptII* and the T-E9 are detected, while *pat* is not detected. The detected targets are present in altogether nine GMOs belonging to one of the three detected species: the authorized GT73 rapeseed, MON863 maize and MON89788 soybean, and the un-authorized Topas 19/2 and MS1 rapeseed, MON87460 maize and MON87705, MON87708 and MON87769 soybean. But Topas 19/2 also contains the *pat* gene, which is not detected. A test is then performed targeting the *ctp2-cp4epsps* construct, which is present in GT73 rapeseed, and in MON 87705 and MON89788 soybean. This test is negative, and the list of un-authorized candidates with the *nptII* is reduced to three: MS1 rapeseed, MON87460 maize and MON87708 soybean (red arrows). Event specific tests targeting these three GMOs are then performed. If these tests are negative, then it must be concluded that the detected *nptII* gene is derived from an authorized GMO, from an unknown GMO (i.e. one not represented in the matrix) or from a non-GM source. Additional ad hoc testing is then required (if justifiable) to determine if the sample contains un-authorized GMO. Scenario 12: The *pat* gene is detected, while the *nptII* gene and the T-E9 are not. The *pat* gene is present in altogether six GMOs belonging to one of the three detected species: the authorized Bt11 maize and A2704-12 soybean and the un-authorized Falcon GS40/90 and Topas 19/2 rapeseed, and A5547-127 and DAS-68416-4 soybean. But Topas 19/2 also contains the *nptII* gene, which is not detected. Event specific tests targeting the other three un-authorized GMOs (blue arrows) are then performed. If these tests are negative, then it must be concluded that the detected *pat* gene is derived from an authorized GMO, from an unknown GMO or from a non-GM source. As for scenario 11, to verify if the sample contains un-authorized GMO will require use of additional tests. Whether this can be justified depends on the specific case, the available resources and the jurisdiction.

the modified trait and genetic elements is probably also available. Currently there are few GMO laboratories that alone have the resources to do this systematically. A more realistic solution would be the organization of a network of collaborating experts that should feed information into a common database or decision support system. A smaller group of experts dedicated to structure the information and make it useful would also be required.

11.1. Fingerprinting techniques

Raymond et al. (2010) and Ruttink et al. (2010a) reviewed the available analytical tools for GMO detection and characterization, and explored DNA fingerprinting as an alternative to identify GMOs. Fragment sizes and fingerprint profiles can be compiled much like the presence/absence profiles of PCR targets described with the matrix approach. Single fragments can also be cloned and used as reference materials, and they can be sequenced to verify if they are of GM origin. Fingerprinting (as well as the use of e.g. immunological tests) can easily be seen as an extension of the more widely applied matrix approach or “molecular toolbox”. Fingerprinting and DNA sequencing may be particularly useful to identify and characterize un-authorized GMOs of ISK-class 3. Once characterized, it is also possible to develop specific PCR modules.

11.2. Microarray technologies

High density microarrays here refer to arrays with thousands of different probes. Tengs et al. (2007; 2010) used such arrays with probes tiled through a compiled set of DNA sequence targets, to try to elucidate if the sample in question contains DNA contig(s) with a high degree of similarity to these targets. A less targeted but related approach was proposed and demonstrated in silico by Nesvold et al. (2005). In this model, the length (*N* nucleotides) of the oligonucleotides on the array was shorter than typical PCR primers and selected from the space of all *N*-mers by in silico subtraction of the *N*-mers present in the corresponding (species) non-GM genome and other *N*-mers unlikely to act as informative evidence of presence of foreign (putative GM insert) DNA. Successively the sequence similarities can be used as anchors for PCR and DNA sequence based characterization.

11.3. Next generation sequencing technologies

The cost of DNA sequencing has dropped dramatically over the last decade and is predicted to continue to drop in the foreseeable future. This can contribute to make use of advanced sequencing technologies more applicable. Transcriptome sequencing is applicable to GMOs expressing novel transcripts only, and is building on the use of subtraction against known non-GM transcriptomes in vitro and in

silico (Tengs et al., 2009). Genomic subtraction has been used to enrich samples for genotype specific sequences for two decades (see e.g. Darrasse et al., 1994). For small genomes, such as those found in microorganisms, it is only a matter of time and available resources before the full genome sequence is determined by so-called next-generation sequencing technologies (Mardis, 2007). Recently this type of technology played a key role in the characterization of the strain of *E. coli* responsible for a high number of deaths in Europe in the spring of 2011 (Mellmann et al., 2011). By subtraction of conserved non-GM segments and re-sequencing of the genome this technology may contribute to the detection of unknown GM materials.

11.4. Prospective of alternatives to PCR

Neither high density microarrays nor next generation sequencing is likely to be used routinely for GMO detection, mainly due to capacity requirements. However, it is reasonable to expect that these technologies in the near future will become cheaper, will require less staff training, and that the necessary equipment will become more widespread. It is therefore likely that alternative and advanced tools gradually will find their way also into the GMO detection arena, at least on an ad hoc basis. An example of such an ad hoc scenario would be if, for a particular product, the perceived risk for adverse effects to health, environment and/or economy is exceptionally high.

12. Discussion and conclusion

It is impossible to give recommendations for a universally applicable testing scheme for the presence of un-authorized GMO. There are simply too many variables involved: scope of the analyses, availability of (validated) analytical tools and e.g. computer assisted decision support systems, information relevant to the sample, the number of plant species in the sample, the competence of the laboratory, etc. Information relevant to the sample or analytical modules can also be “cryptic”.

However, among the several applicable methodologies, the matrix approach is applicable to detecting both authorized and un-authorized GMOs and is de facto in use in numerous GMO testing laboratories. Admittedly, even the matrix approach will probably fail to detect many of the un-authorized GMOs currently under development and some already commercialized or subject to field trials. But there is presently no realistic alternative available that can be applied routinely.

It is recommended that laboratories with little or no experience with standardized screening analysis and results interpretation do not apply ad hoc testing schemes. This is because of the very broad spectrum of possible scenarios, outcomes and sources of error, misinterpretation and analytical uncertainty. We recommend that laboratories begin with the application of well characterized and validated screening and event specific modules used by other more experienced laboratories. This will increase harmonization and facilitate dialog and recognition of strengths and weaknesses of applied concepts and approaches; and also of results interpretation and decisions on how to proceed on the basis of initial screening results. Considerations on costs and time, availability of information on the origin of the sample, the cultivations, field-trials and developments of GMO in different countries, reference materials and validated PCR modules, etc. are relevant. The complexity of the problem may restrain the laboratory from making the optimal choices, and decision support tools are therefore desirable. Examples of such tools include the GMOTrack (Novak et al., 2009), the GMOseek (unpublished data) and the Co-Extra DSS (Bohanec et al., 2013).

Unintended escapes and commingling of un-authorized GMOs can never be ruled out (GAO, 2008). With the expected rapid increase in the number of GMOs that cannot be detected with the currently available screening tools, there is an evident need for development of new

analytical tools and strategies. Globalization of trade is unlikely to be reversed. Even the USA that previously did not need to consider the possibility that un-authorized GMOs could be imported now acknowledge this possibility and the need for appropriate measures (USDA, 2008).

Technology will continue to develop, as will the body of characterized genes and genomes available to developers of GMOs. Most likely there will also be a growing number of GMOs with traits of industrial or pharmaceutical relevance but not intended for food or feed use. These could also enter the agricultural supply chains and cause ethical and religious concern or even pose a significant risk to human and animal health. It is of utmost importance that producers and other stakeholders maintain segregation of supply chains as the public controls will only be able to reveal contamination a posteriori. The GMOs detectable with a matrix approach based screening strategy are primarily what we classified as the first and second generation of GMOs. Third and fourth generation GMOs will require much more specific analytical modules and possibly also detection methods other than PCR. If first and second generation GMOs are phased out quickly, there will soon be few GMOs left that are detectable with this approach. Third and fourth generation GMOs will sooner or later outnumber the first and second generation GMOs. Even if the number and diversity of targets for screening modules is increased significantly, inevitably reliance on screening alone will never be able to ensure the detectability of all relevant GMOs. Thus, reliance on screening can potentially mislead stakeholders.

There are many pitfalls that can lead to wrong conclusions. Roughly, one can distinguish between false positives (type I error), false negatives (type II error) and quantitation errors. Each of these can have severe consequences for trade, safety and trust in regulators, industry, etc. Wrong assumptions about the specificity and performance of individual testing modules are perhaps the two most important factors causing error. The list of GMOs containing a particular element or construct specific target is often incomplete due to limited access to relevant information. There are many cases where the presence of a particular GMO is non-compliant with legislation or a trade contract. When interpretation of screening results indicates presence of such a GMO we strongly recommend verification using an event specific PCR module or DNA sequencing.

The presence/absence in a particular GMO of the target of a PCR module should be established experimentally, if at all possible. Theoretical presence of a target must be treated with caution, as the target may be truncated, rearranged, exhibit substitutions in primer/probe loci or even be absent in some cases. As pointed out in Section 10.2 the use of quantitative data can aid the analyst, but particular attention should be given to results at near LOD concentrations and comparability of amplification efficiencies. The latter can vary significantly for similar targets in different GMOs, but must be nearly equal throughout the PCR to provide reliable quantitative estimates (Cankar et al., 2006; Nogva and Rudi, 2004).

We believe that both copy numbers, sizes of inserts/target fragments and complete DNA sequence data could be added to the matrix as additional useful information. However, this cannot be a requirement. Large reference databases linking molecular data and other information with the specific analytical modules can undoubtedly contribute to improve the reliability of analytical work and results interpretation. The main challenges in this respect are the coordination of data input and quality assurance and how the data can be made available to the analytical laboratories.

Perhaps the most important single factor responsible for the lack of reliable approaches to comprehensive detectability of un-authorized GMOs is the lack of global transparency. The Biosafety Clearing House (BCH) and the Cartagena Protocol on Biodiversity (CPB) and corresponding databases are instruments that shall play a pivotal role in this respect. However, not all countries have ratified the protocol yet and others do not feed in the appropriate information

regarding GMO developments and field trials to the BCH. Perhaps the situation will change as a consequence of a possible emergence of unauthorized GMOs onto the market in these countries. Davison (2010) speculated that convergence of interests could reassemble e.g. the EU and USA in an unexpected way.

Considering the size and severity of the problems that unauthorized GMOs could potentially represent, we find it timely to call for: 1) increased awareness among the relevant stakeholders, and 2) implementation of effective segregation routines.

Company stewardship is crucial and guidelines such as the USDA Biotechnology Quality Management System (BQMS; USDA, 2011) are useful in this respect. Development of new detection tools takes several years, the validation of the standard methods even more. Several years of “immobility” in developing robust new tools to trace unknown GMOs routinely have passed by. Now, it is time to upgrade the level of GMO detection to a level matching the discriminating capacities required by the new generation of GMO to ascertain and safeguard the quality of all products in the food/feed chain globally.

Acknowledgments

This review has partly been made possible as a result of the EU-funded integrated project Co-Extra <http://www.coextra.eu> (contract FOOD-2005-CT-007158) and discussions within an ad hoc working group of the European Network of GMO Laboratories (ENGL) over several years. AHJ is grateful for financial support from the Norwegian Research Council (grants 170363 and 178288). MP is grateful for financial support from the Spanish Ministerio de Ciencia e Innovación (project AGL2010-17181). The authors are grateful to numerous colleagues including colleagues from outside Europe for contributing to these discussions. The following colleagues from countries outside the EU are acknowledged for contributing specific information relevant to the preparation of this review: Nelly Datukishvili, Chris Viljoen, Kazumi Kitta, Hiroshi Akiyama, Jasbeer Kaur, Nilson Cesar Castanheira Guimaraes, Luciana Pimenta Ambrozecius, Marcus Vinicius Segurado Coelho. Without global cooperation it will never become possible to develop effective systems to limit the presence of un-authorized GMOs in the agricultural supply chain.

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